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Laboratory Investigation

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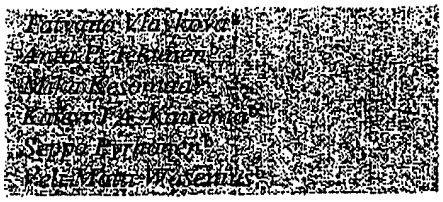

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Increased Thymidylate Synthase Gene Expression in Metastatic Melanoma

Key Words
Metastatic melanoma
Thymidylate synthase
Gene expression

Abstract
Thymidylate synthase (TS) provides the only de novo source of thymidylate for DNA synthesis and is a key target for cancer chemotherapeutic agents. We investigated the TS gene expression by semiquantitative reverse-transcriptase polymerase chain reaction in metastatic melanoma and compared the results with those from control tissues. The relative TS/β-actin level ratios were 0.5, 0.9, 0.3, 0.4, and 0.5 (mean 0.5) in skin, lymph node, thyroid, muscle, and spleen, respectively. In metastatic melanoma samples, the ratios varied from 0.9 to 2.7 (mean 2.0). The differences of expression levels between these two groups of samples were statistically highly significant ($p = 0.0000713$). A similar statistical significance ($p = 0.0002$) was observed between patients achieving a complete response and patients who had progressive disease despite immunochemotherapy. There was no clear relationship between a high TS/β-actin ratio and the S phase fraction, as all melanomas had a high S phase fraction.

Introduction

The goal for the development of treatment in disseminated melanoma is to generate a high frequency of long-term complete responses (CRs). Although we can achieve responses of 60% in cases treated by our regimen, only 10% of these responses are prolonged CRs. The treatment is intensive and has several side effects [1]. Thus, the selection of patients likely to get CR would improve the overall results considerably, while patients unlikely to respond could be treated by another treatment scheme. The occurrences of durable CRs in metastatic melanoma treated by interferon have been related to population shifts in blood T cell subsets during therapy [2, 3]. To date, no other clear correlation with regard to CR between interferon combination chemotherapy and any biochemical factor has been reported in metastatic melanomas.

Thymidylate synthase (TS; 5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) is essential in the de novo synthesis of thymidylate, a precursor of DNA. Expression of its activity is strongly dependent on the cell cycle. It is important for understanding the abnormal growth of cancer cells as well as for cancer chemotherapy to elucidate the expression of the TS gene and to evaluate its prognostic value in clinical practice.

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Table 1. Characteristics of metastatic melanoma samples

Sample No.	Patient age/sex	Primary site	Therapy	Response	Site of metastasis	Follow-up, months/status
1	43/female	subcutis, left breast	post	CR	local, breast	12+/NED
2	41/female	skin, lower extremity	post	PD	lymph node	14/DE, DM
3	67/female	NK	post	CR	lymph node	25/DE, DM
4	41/female	skin, upper extremity	post	CR	local, left arm	43/LRP
5	60/female	skin, chest	prior	PD	local, chest	10/DE, DM
6	70/female	skin, eye lid	post	CR	local, orbita	54+/NED
7	67/male	skin, lower extremity	prior	PD	local, subcutis	27/DE, DM
8	71/female	skin, lower extremity	post	PD	multiple	18+/DM

NK = Not known; post = tumor samples were taken after chemotherapy; prior = tumor samples were taken before chemotherapy; CR = complete remission; PD = progressive disease; NED = no evidence of disease; DE = dead; DM = distant metastases; LRP = local recidive or progression.

The TS is a housekeeping gene which is expressed at much higher levels in proliferating cells than in quiescent cells. TS introns contain sequences that are necessary for normal growth-regulated expression of the mouse TS gene [4]. These sequences appear to be associated with sequences that are important for splicing and function in cooperation with upstream regulatory elements to bring normal S-phase-specific expression. The complete nucleotide sequence of the human TS gene has been determined by Kaneda et al. [5]. The biologically active unit spans about 16 kilobase pairs and is composed of seven exons and six introns.

Patients with metastatic melanoma have still a poor prognosis, the mean survival being 6 months [6]. By multifactor analysis, the most significant prognostic variables are the number of metastatic lymph nodes, the presence of ulceration, patient's age, and extent of the primary tumor [6]. As a consequence of constant chemotherapy failures, better biochemical prognostic factors than currently available are widely searched. The tumor-proliferative rate measured by the DNA S phase fraction and DNA ploidy is known to be related to the chemotherapeutic response in melanoma [7], and cell cycle characteristics have also been evaluated as favorable prognostic factors [8, 9].

Here, we present our results of TS mRNA expression in human malignant melanoma both in metastatic and primary lesions as well as in control tissue samples. We also relate our findings to the clinical context of each case of metastatic melanoma and discuss the clinical role of TS measurements.

Materials and Methods

Patient and Tumor Characteristics

We studied eleven frozen metastatic melanoma tumors from 8 patients treated at the Department of Oncology of the Helsinki University Central Hospital. The samples analyzed were taken between July 1989 and December 1993. The patient population included 7 women and 1 man, all aged from 41 to 71 (median 60) years (table 1). The primary sites of metastatic melanoma included the skin on an extremity in 4 cases and the skin of other sites in 2 patients; one deep subcutaneous melanoma was also included (sample 1); in 1 case (sample 3), the origin of the primary tumor remained unknown (table 1). The site of metastasis varied considerably including lymph nodes in 2 patients (samples 2a and 3) and skin and subcutis in 5 patients (table 2). Multiple metastases were recorded in 1 patient (sample 8). All metastatic melanoma patients received chemotherapy consisting of dacarbazine, vincristine, bleomycin, and lomustine combined with natural leukocyte interferon [1]. Tumor samples were taken after chemotherapy in 2 patients (samples 5 and 7). The remaining 6 patients got chemotherapy after tissue sampling. Four patients had CRs and 4 patients had progressive disease (PD) despite the treatment. Two patients are still alive and well without any evidence of disease (samples 1 and 6). The median follow-up time is 25 (range 10–54) months.

Tissue Samples

The samples were obtained from patients treated at the Department of Oncology, Helsinki University Central Hospital. The samples were frozen immediately in liquid nitrogen and kept at −80°C after surgical excision. All specimens were inspected by a pathologist to identify the type of tissue and confirm histological diagnosis and origin. We analyzed 20 tissue samples, including 11 metastatic melanoma tissues from 8 patients, 3 primary cutaneous melanomas, and 6 control samples representing different tissue origins: skin, lymph node, thyroid, spleen, and muscle (table 2). Normal skin and lymph nodes were chosen for reference tissues, because most of the metastasis sites were in these types of tissues. Other tissues were chosen to represent major organ distribution. Special care was taken to use tissues obtained from patients not having a malignancy.

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Table 2. Origin, TS/β-actin ratio, S phase, and ploidy of tissue samples

Sample No.	Site of sample	TS/β-actin ratio[a]	S phase %	DNA index
Metastatic melanoma samples				
1	left breast	2.4	18.9	1.65
2a	lymph node	1.4	8	1.0
2b	skin	1.9	7.1	1.0
3	lymph node	2.5	15.9	1.82
4a	skin	2.7	13.9	1.88
4b	skin	2.3	n.d.	n.d.
5	skin	1.8	29	1.82
6	orbita	2.6	24	1.65
7a	skin	1.5	16.5	1.49
7b	skin/subcutis	1.5	18	1.55
8	skin/subcutis	0.9	17.8	1.95
Primary melanoma samples				
9	primary	2.0	n.d.	n.d.
10	primary	2.5	n.d.	n.d.
11	primary	3.1	n.d.	n.d.
Normal-tissue samples				
12	skin	0.5	n.d.	n.d.
13	lymph node	0.9	n.d.	n.d.
14	spleen	0.5	n.d.	n.d.
15	thyroidea	0.3	4.3	1.0
16	muscle	0.4	n.d.	n.d.
17	muscle	0.4	1.5	1.0

n.d. = Not done.

[a] Calculated from densitometric data obtained from ethidium bromide electrophoresis.

To avoid selection bias, we tested all samples from patients achieving CR or PD existing in our tissue bank. In addition, internal methodological bias was prevented by separating the scoring and calculations totally from the collection of clinical data.

RNA Isolation and Reverse-Transcriptase (RT) Reaction

Total RNA was isolated by the guanidium isothiocyanate procedure [10]. The RNA concentration was estimated by measurement of the optical density at 260 nm, and the quality was evaluated by agarose gel electrophoresis with 1% formaldehyde. cDNA was synthesized using 0.2 μg of random hexadeoxynucleotide primers, 5 μg of total RNA as a template, and 5 U of Maloney murine leukemia virus reverse transcriptase (M-MuLV Reverse Transcriptase; first-strand cDNA synthesis kit, Pharmacia). After incubation at 37 °C for 1 h, the reaction mixture was stored at −20 °C.

Polymerase Chain Reaction (PCR) Amplification

For PCR amplification, the primer sequences were selected from different exons of the β-actin and TS genes as follows: β-actin sense, 5′-CGGGAAATCGTGCGTGACAT (corresponding to the bases 2107–2126) [11, 12]; β-actin antisense, 5′-GGAGTTGAAGGTAGTTTCGTG (bases 2429–2409) [12]; TS sense, 5′-AGATCCAACACATCCTCCGCT (bases 107–127) [12, 13]; TS antisense, 5′-CAGAACACACGTTTGGTTGTCA (bases 242–221) [12, 13]. Both primer pairs were selected so that the genomic DNA contained an intron between the PCR primers. Thus a contamination of genomic DNA could be easily separated by size.

To minimize the effect of the variation in amount of initial template, TS and β-actin genes were coamplified in the same tube. Linear amplification regions were determined by serial dilutions of the cDNA sample [12]. TS primers were added at the beginning of the reaction and β-actin primers after 10 cycles, as it was preferred to amplify both low-abundance TS mRNA and high-abundance β-actin in linear PCR regions and to obtain bands sufficiently intensive on agarose gel from all samples. The cycle number of ten was a compromise between optimal cycle numbers for melanoma and control samples. These were 37 for TS and 23 (controls) and 27 (melanomas) for β-actin, respectively.

An aliquot of 4 μl from six to eight dilutions of each cDNA reaction product was heated up to 95 °C for 5 min and used as a template for PCR amplification with 20 pmol of primers, 1 × PCR buffer (50 *mM* KCl, 10 *mM* Tris-HCl, pH 8.3, 1.5 *mM* $MgCl_2$), 1 μl of 20 *mM* dNTPs, and 2.5 U of Taq polymerase (Pharmacia). The final volume was adjusted to 50 μl water. Denaturing was at 95 °C for 60 s, annealing at 55 °C for 90 s, and polymerization at 72 °C for 60 s, for altogether 30 cycles.

Quantification of PCR Amplification

Electrophoresis of 10 μl of the PCR reaction mixture was performed in a 2% agarose gel followed by staining with ethidium bromide. This method was routinely used for initial screening and was fast, easy, and economical. The size ladder was from 24 to 726 base pairs (Phi X 174; Promega, Madison, Wisc., USA). To quantitate the PCR product, negatives from the photographs (Polaroid 665) of the gels were evaluated densitometrically (Hoefer Scientific Instruments, San Francisco, Calif., USA). The amount of TS product was normalized to the amount of β-actin. Densitometric data were further assessed when three samples with ten to twelve dilutions from each were studied by 'hot PCR' using 0.25 μl [α^{32}P]dCTP (3,000 Ci/mmol; Amersham, Little Chafont, UK) per reaction. Gel electrophoresis was performed as described earlier and dried on nylon filter (Hybond-N, Amersham). Besides normal autoradiography, we performed a digital autoradiography to get more distinct quantification of the PCR products. The specimens were incubated on a high-resolution photostimulable plate (Fuji-III; Fuji, Tokyo, Japan) and read by an image reader (Digiscan; Siemens, Erlangen, Germany) using a linear fixed scale program. The images were saved on an optical disk in a VAX-hosted (Digital Equipment) image workstation. The images were then copied to a Macintosh computer (Apple Computer, Cupertino, Calif.) through Ethernet and analyzed with NIH Image 1.55, a public domain program from the National Institutes of Health (Bethesda, Md., USA).

Calculation of Gene Expression Ratios

PCRs of β-actin and TS were run in one tube, in order to eradicate any error due to different amounts of starting template. Dilution curves for the genes were created by running simultaneously both PCRs using different cycle numbers to determine linear regions for each PCR. The optimal amount of cDNA was determined using points on the linear part of the β-actin curve. TS/β-actin ratios were calculated from two, three, or four points. We used equal conditions

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for melanoma and control samples, ensuring comparable results. Data were statistically analyzed by the Student t test.

Flow Cytometry

Tumor samples for flow cytometry were frozen in liquid nitrogen in citric buffer containing 5% dimethyl sulfoxide [14] and stored at -80°C. At the time of analysis, the samples were rapidly thawed in a 37°C water bath and mechanically disaggregated and filtered through a 50-μm nylon mesh. The cell suspension was centrifuged for 5 min at 1,600 rpm, resuspended into 0.5 ml of propidium iodide (50 μg/ml in 10 m*M* Tris buffer, 1 m*M* EDTA, 0.3% Nonidet P40, pH 7.5), and incubated on ice for 15 min. Then 0.25 ml of RNase (1 mg/ml; Sigma, St. Louis, Mo., USA) was added for 15 min at room temperature. The sample was filtered through a 30-μm nylon mesh immediately before analysis. Human lymphocytes were used as external ploidy standards and chicken and salmon erythrocytes as internal reference standards.

The nuclear suspensions were analyzed with an EPICS C flow cytometer (Coulter, Hialeah, Fla., USA) with 488-nm argon ion laser excitation. The instrument was calibrated with fluorescent beads, and at least 10,000 nuclei per sample were analyzed and the total emission above 590 nm measured. The DNA index in samples with DNA aneuploidy was calculated as the ratio of the aneuploid stem line G_1 peak channel to diploid stem line G_1 peak channel. The S phase fraction was calculated based on the assumption that the S phase fraction is the rectangle between the G_0/G_1 and G_2/M phases of the cell cycle. Counts per channel in the mid-S phase (10 channels) were calculated and multiplied by the number of channels between the G_0/G_1 and G_2/M peaks to obtain the total S phase fraction.

Results

Reproducibility of the Method

To test the reproducibility of the system, PCR runs were repeated from four cDNA samples, and quantitation was done as described earlier. Calculated ratios were as follows: 2.6 and 2.8 for sample 4a, 3.1 and 3.1 for sample 11, 1.5 and 1.5 for sample 7a, and 2.1 and 1.5 for sample 5. Thus, the average difference between repeats was only 5.1% as calculated from the mean value.

Dilution curves with wide linear region were made for all samples (fig. 1). The validity of the system was checked by performing PCR repeatedly, using cDNA from different RNA extractions. A high overall reproducibility was achieved in the melanoma and muscle samples studied. The ratios were 0.5 and 0.3 for the control sample and 2.4 and 2.2 for the melanoma sample.

Enhancement of Densitometry by Digitalized Data Managing

'Hot' RT-PCR was run from one skin control (sample 12) and two melanoma tissues (samples 4a and 11) to further improve the quality of densitometry by minimizing of the gel background. We performed a digital autoradiography besides ethidium bromide electrophoresis and normal autoradiography (fig. 2).

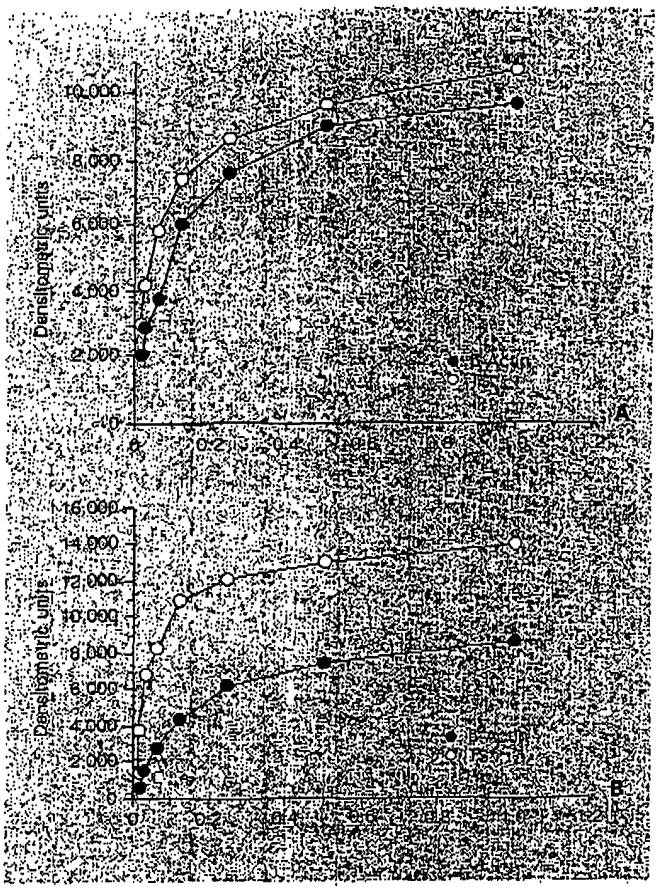

Fig. 1. Dilution curves of TS and β-actin PCR. **A** Data points obtained from sample 2a. **B** Data points from sample 3. The calculated TS/β-actin ratios are 1.4 and 2.5, respectively. The ratio is calculated from densitometric data obtained from ethidium bromide electrophoresis.

Measurements of the TS/β-Actin Ratio

The relative gene expression ratios of TS and β-actin were 0.5, 0.9, 0.3, 0.4, 0.5, and 0.4 in skin, lymph node, thyroid, muscle, spleen, and muscle, respectively (table 2). The mean value for all controls was 0.5. Primary melanomas had ratios from 2.0 to 3.1. The ratios in metastatic melanoma samples varied from 0.9 to 2.7. There were only three samples from 2 patients failing the therapy (PD) in whom the samples were taken before chemoimmunotherapy: 2a, 2b, and 8, the TS/β-actin ratios being 1.4, 1.9, and 0.9 and S phase 8, 7.1, and 17.8%,

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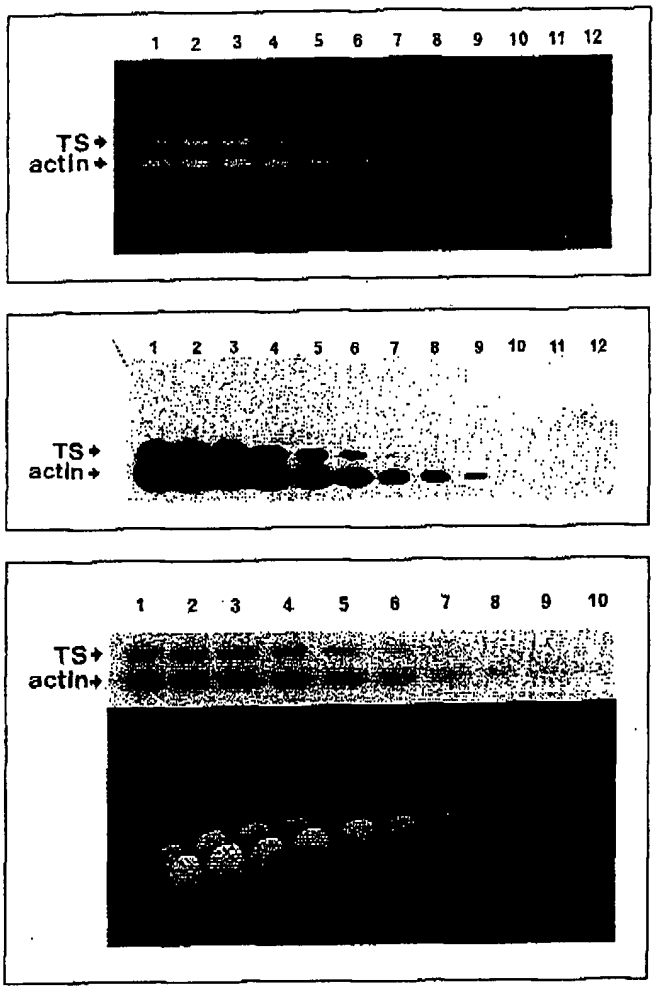

Fig. 2. A Ethidium bromide gel electrophoresis staining (sample 4a). Various lines (1–12) represent PCR products from diluted cDNA samples (dilution factor 1/2). We used ordinary 2% agarose gel with ethidium bromide staining followed by densitometry to obtain the data presented in table 2. **B** Electrophoresis of 'hot' PCR (sample 4a, see **A**). In order to sensitize the PCR, a radioactive label was used in the PCR reaction mixture, and an autoradiogram was made from a gel dried on a nylon membrane. **C** Digitalized autoradiogram of sample 4a (see **A**). To further investigate the possibility of minimizing the effect of background and enhancing the band separation, a digitalized autoradiogram was performed. Upper panel: original autoradiogram on plain film; lower panel: digitalized autoradiogram as surface plot presentation on a linear digital scale.

respectively. All CR samples were taken before chemoimmunotherapy and had higher TS/β-actin ratios (2.3–2.6) and higher S phase (13.9–18.9%). All PD samples taken after the chemoimmunotherapy had a high S phase (16.5–29), but a low TS/β-actin ratio (1.5–1.8).

Statistical Significance

Data were analyzed by the Student t test and showed a significant difference between the ratios of metastatic melanoma and control samples ($p = 0.0000713$) and primary melanoma and control samples ($p = 0.0000603$), but not between metastatic and primary melanoma samples ($p = 0.127$). If we compare all CR samples to PD samples, the TS/β-actin ratios were statistically different ($p = 0.0002$). The number of cases with different responses where the samples were taken prior to therapy does not allow us to draw any definite conclusions about the prognostic significance of the TS analysis, but we can conclude overall that there was no statistical significant difference between samples taken prior to and after therapy ($p = 0.239$).

S Phase Fraction and Ploidy

Flow cytometric data were obtained from ten metastatic melanoma samples (table 2). The mean value of S phase fractions for patients achieving CR was 18.2 (13.9–24), and the mean of the patients having PD was 16.1 (0.9–29). No statistical significant difference was obtained ($p = 0.32$). The tissue materials of primary melanomas were not large enough to perform retrospective flow cytometry. Two control samples, muscle and thyroid tissue, were also analyzed retrospectively.

Discussion

Our preliminary study is the first report to our knowledge suggesting a relationship between increased TS expression and favorable treatment response in metastatic melanoma in vivo. TS/β-actin ratios were higher in all melanoma samples from CR patients than from those patients with PD (tables 1 and 2). It seems that a high TS mRNA level occurs in melanomas sensitive to our chemoimmunotherapy, while a low TS mRNA level predicts resistance for the same regimen. Our results suggest that further studies with more patients and probably at the protein TS level are needed to show whether TS levels may be used as a determinant for selecting patients in treatment regimens.

In all cases of metastatic melanoma, except sample 2, the S phase was high irrespective of CR or PD, indicating that treatment responses were not related to cell cycle measurements. Previously, Elledge et al. [15] have demonstrated higher TS mRNA levels in 5-FU-sensitive than in 5-FU-resistant breast cancer cell lines. On the other hand, the expression of TS was found to be a strong prog

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nostic factor for resistance in non-small-cell lung carcinomas, as shown by a progressive clinical course and a short survival of the patients [16]. The same is true for ovarian cancers treated using the 5-FU-leucovorin and the 5-FU-cisplatin combinations, since the low TS expression in tumors was related to frequent treatment responses [12]. One reason for the controversy between these results and ours is probably the different cancers studied and differences in the therapy used. Instead of 5-FU, we used decarbazine, vincristine, bleomycin, and lomustine combined with natural leukocyte interferon [1]. In the current study, the highest TS/β-actin ratios were measured in samples of patients who achieved CR from interferon combination chemotherapy. Significantly lower ratios were obtained in PD samples, as demonstrated in table 2. If we place a cutoff value of 2.0 for the TS/β-actin ratio, all CRs are above and all PDs and controls are below that value. This suggests that the ratio may serve as a prognostic factor. The TS/β-actin ratio of 2.0 accepted as a cutoff point was chosen because it appeared to be the mean value of all samples studied, including normal and primary melanoma, and was close to the other mean value, calculated on the basis of all metastatic melanoma samples studied.

In our study, the TS/β-actin ratios in primary melanomas were high; however, in control samples, the ratios were low. The relative amount of TS was only increased 2- to 3.1-fold, and this may not be enough to confer any resistance type situation. Our results are not in contradiction to results published earlier by other authors [12, 17], although these have reported that increases of TS in the 10- to 100-fold range are associated with drug resistance. In addition, gene amplification has been shown to result in TS overproduction and in vivo resistance to 5-FU [18]. Other factors may play a role in determining the drug sensitivity in melanoma tumor samples, e.g., the status of functional signal transduction pathways and of repairing mechanisms. Additionally, a high TS level was found to be a favorable prognostic factor in colorectal cancer [19].

Based on our experience using dilution curves of RT-PCR, we consider the PCR method only semiquantitative rather than absolute in gene expression. We found a high reproducibility of RT-PCR, as demonstrated in subsequent repeats from two different batches of RNA extractions from the same sample. This was done in one metastatic melanoma (sample 4b) and one muscle (sample 17) tissue. Furthermore, it appeared that there was no significant benefit of making further technical assessments, as 'hot' PCR and digitalized autoradiographs gave similar results as ethidium bromide staining followed by densitometry. However, the linearity scale (fig. 2B) seemed to be wider, as seen in the increase of the number of bands to be analyzed as compared with figure 2A. This could mean a significant advantage when measuring low-abundance transcripts. Because of the relatively high abundance of transcript and the complexity of producing digitalized results, we based our screening results in this study on ethidium bromide staining. Nevertheless, our results indicate that the digital autoradiography method gives reliable and reproducible results which were in accordance with those obtained by ethidium bromide staining. Digital autoradiography can be easily performed in institutions with the possibility to utilize digitalized roentgenography. Linearity of the calibration curves and programming of photostimulable plate responses have to be tested by serial dilutions before absolute quantification. The absolute 'hot' PCR activities in each band can be calculated, and this may provide further advancements RT-PCR analysis.

We conclude that semiquantitative RT-PCR analysis offers the advantage of reliably detecting TS mRNA levels from small amounts of starting material. Each sample should always be analyzed separately with individual determination of optimal PCR. Semiquantitative TS levels, TS/β-actin ratios over 2.0, were found to relate to the CR achieved by an immunochemotherapy regimen. Even with this small number of patients we were able to produce statistically relevant conclusions. In any case, these preliminary results justify further studies involving a greater number of patients and different regimens.

Acknowledgement

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